

CONCORDANCE AND CONTROVERSY IN DETERMINING THE OMEGA-3 INDEX IN PLASMA AND RED BLOOD CELLS MEMBRANE

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Abstract

Omega 3 fatty acids present immunomodulatory properties demonstrated by numerous studies. Accurate determination of fatty acids (FA) in different biological matrices is of special physiopathological and clinical value. The aims of our study were to develop a LC/MS method for the determination of main FA in plasma and red blood cell membranes (RBC) and to establish a possible correlation between the amount of omega 3 acids in plasma and in the membrane of RBC. Determinations were performed on a Kinetex C18 column (50 x 3 mm, 2.6 μ m), using an isocratic mixture of acetonitrile: 0.2% acetic acid in water as the mobile phase, at a flow rate of 0.4 mL/min. The method permitted the separation of nine FA, plasma concentration and, respectively, RBC membrane values were used for the calculation of parameters (ratios) of clinical interest. The existence of a correlation between plasma levels of certain FA and their incorporation into RBC membrane was determined on 26 blood samples. The study showed a significant difference ($p < 0.001$) between plasma concentrations /RBC membrane values, and on the other hand, that there is a correlation between the AA/DHA ($r = 0.7540$) and AA/(EPA + DHA) ($r = 0.7807$) ratio value in plasma and RBC membrane and a weak correlation between plasma levels of AA/EPA ratio ($r = 0.3906$) or Omega-3 Index ($r = 0.4991$) and the FA levels into the RBC membrane.

Rezumat

Acizii grași omega 3 prezintă proprietăți imunomodulatoare demonstrate de numeroase studii. Determinarea cu acuratețe a acizilor grași în diverse matrici biologice prezintă o importanță fiziopatologică și clinică deosebită. Studiul nostru propune o metodă LC/MS de determinare a acizilor grași din plasmă și membrana hematiei, precum și verificarea existenței unei corelații între valoarea plasmatică a acizilor omega 3 și încorporarea lor în membrana eritrocitară. Determinările s-au efectuat pe o coloană Kinetex C18 (50 x 3 mm, 2,6 μ m), folosind ca și fază mobilă un amestec izocratic de acetonitril: 0,2% acid acetic în apă, cu un debit de 0,4 ml/min. Metoda a permis separarea a 9 acizi grași, iar valorile de concentrație calculate pentru aceștia în plasmă și, respectiv, membrana eritrocitară au fost folosite pentru calcularea unor parametrii (rapoarte) de interes clinic. Verificarea existenței unei corelații între valorile plasmatică ale unor acizi grași și încorporarea lor în membrana eritrocitară s-a efectuat pe 26 de probe de sânge. Studiul a arătat că există o diferență semnificativă ($p < 0,001$) între valorile de concentrație plasmă/membrană, iar pe de altă parte că există o corelație între valoarea raportului AA/DHA ($r = 0,7540$) și AA/(EPA + DHA) ($r = 0,7807$) în plasmă și membrana eritrocitară și o corelație slabă între valorile plasmatică ale raportului AA/EPA ($r = 0,3906$) sau a indicelui omega 3 ($r = 0,4991$) în momentul recoltării și proporția lor în membrana eritrocitară.

Keywords: Omega-3 Index, omega 3 fatty acids, LC/MS

Introduction

The most important omega 3 polyunsaturated fatty acids (n-3PUFA) and omega 6 (n-6PUFA) - eicosapentaenoic acid (C20:5n-3) (EPA), docosahexaenoic acid (C22:6n-3) (DHA) and arachidonic acid (C20:4n-6) (AA) originate either from food sources (fish and marine fish oil for omega-3 series and respectively, vegetal oils and fats for the omega 6 series) or *in vivo* by carbon chain elongation of parent compounds - linoleic acid (C18:2n-6) (LA) and α -linolenic acid (C18:3n-3) (ALA) [14]. Modern

human diet rich in products of vegetal origin limits the intake of n-3PUFA because the plants do not convert C18n-3 to C20 or C22 and a part of ALA enters the β -oxidation in human body [15]. Literature data show that an increased intake of PUFA either through diet or supplemented by medication, and especially n-3PUFA with anti-inflammatory properties, lower blood pressure, serum triglycerides and reduce cardiovascular risk [8]. Plasma ratio n-6PUFA/n-3PUFA is not always conclusive [17] and the total blood ratio of EPA/AA presents high variability [19]. Because of less

sensitivity to recent intake and a slower turnover rate, RBC membrane FA may be superior to plasma composition for long-term homeostasis of these compounds [13]. Since a correlation between the amount of PUFA in the membrane of different cells of human body and RBC was established, minimally invasive procedures for determination of the n-3PUFA proportion to the rest of membrane FA (omega-3 Index) are more and more used [2].

Omega-3 Index is defined as the percentage of n-3PUFA (EPA+DHA) of total FA in RBC membrane [12]. Use of RBC membranes for Omega-3 Index calculations ensures patient compliance for biological sampling and a relatively simple sample preparation for analysis in comparison with other types of biological samples. Literature data describe LC/MS or gas chromatography methods for n-3PUFA determination from biological samples or Omega-3 Index, consequently the purpose of our study was to determine whether there is a profile of plasma n-3PUFA reflecting their incorporation into the RBC membrane [1, 6, 16].

Materials and Methods

Standards, reagents and plasma sampling

The standards of purity at least 98% were purchased as follows: EPA, DHA, lauric acid (LAU), LIN, adrenic acid (ADR), oleic acid (OL) from Cayman Chemical Company, palmitic acid (PAL), AA, stearic acid (STE) from Sigma-Aldrich. Internal standard, ketoprofen (KET) was purchased from European Pharmacopoeia. All solvents were of HPLC grade and water was obtained from a Millipore Simplicity Milli-Di purification system. Blank plasma for calibration standard preparation was obtained from the local transfusion centre.

Plasma samples were collected from volunteers entering the for routine laboratory analysis, after obtaining their informed consent. Blood was collected into anticoagulant tubes (heparinised) and plasma separation was immediately carried out by centrifugation for 10 minutes at 2000 g. The plasma samples were stored at -20°C until analysis.

Preparation of plasma samples and standards for analysis

Plasma samples from 26 volunteers were prepared as follows: 0.2 mL plasma were added to 20 µL methanol, 20 mL KET of 100 µg/mL, followed by vortex stirring, 50 µL HCl 10% were then added and stirred at the vortex again. Fatty acids were extracted in 2 mL hexane, and the residue of evaporated organic phase was dissolved in 200 µL acetonitrile. Plasma standards were obtained from blank plasma with added standard solutions followed by extraction as plasma samples. Internal standard singlicate quantification method was performed based on peaks area.

Hydrolysis of RBC membrane phospholipids and FA extraction

A volume of 1 mL of blood (collected in heparin) was centrifuged for 10 minutes at 400 rpm. After centrifugation the white blood cells separate above the RBC layer and plasma was then separated. The RBC were washed 3 times with a washing aqueous solution (pH = 7.4) containing NaCl, HEPES, glucose, bovine albumin. The RBC lysis solution containing phosphate and EDTA pH = 8 was added and centrifuged to remove the supernatant until it is colourless (total elimination of haemoglobin). A volume of 1 mL of HCl 0.5N was added to the RBC membranes and left for 12 hours at 70°C in a water bath. After cooling, the sample was treated with 1 mL 5% NaCl to ensure separation of HCl from the organic phase and stirred at the vortex. The organic phase was then extracted in 2 mL of hexane, concentrated by evaporation and resumed in 200 µL acetonitrile. Samples were extracted in the same day and stored at -20°C after organic phase evaporation until the day analysis.

Standards for fatty acids quantification in RBC

Determination of FA obtained by hydrolysis of RBC membrane phospholipids was performed with a mixture of standard samples containing 100 µg/mL of STE, EPA, LAU, LIN, 400 µg/mL AA, 250 µg/mL OL and 10 µg/mL ADR, and DHA. The standard mixture was incubated for 12 hours at 70°C in a water bath with 1 mL of HCl 0.5N, and 1 mL 5% NaCl was added. The organic phase was then extracted in 2 mL of hexane, concentrated by evaporation and resumed with 200 µL acetonitrile. Extraction efficiency was determined by analysis of a sample with expected concentrations.

The chromatographic system

The Agilent 1200 HPLC Series system (Agilent Technologies, Santa Clara, USA) consisted of the following components: autosampler, binary pump, column oven, thermostat and degasser. The compounds were separated on a Kinetex C18 (50 x 3 mm, 2.6 µm) column under isocratic conditions using a mobile phase consisting of an isocratic mixture of acetonitrile and acetic acid 0.2% in water 80:20. The flow rate was 0.4 mL/min. The column temperature was 40°C, while the autosampler was maintained at 20°C. The detection was performed by an Agilent 1100 LC-MSD Trap system with the following parameters set: ESI negative mode; nebulizer gas, 60 psi; dry gas, 12 L/min; dry temperature, 350°C. Chromatograms were processed using QuantAnalysis 5.2 for LC MSD Agilent Technologies software.

Results and Discussion

The developed analysis method allows the determination of 9 FA in plasma or RBC samples.

The monitoring ions (Figure 1) and retention times (Figures 2 and 3) were as follows: $m/z = 199$ at $R_t = 1.4$ min for LAU, $m/z = 257$ from 301 at $R_t = 2.0$ min for EPA, $m/z = 283$ of 327 for DHA at $R_t = 2.5$ min, $m/z = 259$ from 303 at $R_t = 2.7$ for AA, $m/z = 279$ at $R_t = 3.1$ min for LIN, $m/z = 255$

at $R_t = 4.3$ min for PAL, $m/z = 287$ from 331 at $R_t = 4.3$ min for ADR, $m/z = 281$ at $R_t = 4.7$ min for OL and $m/z = 283$ at $R_t = 8.2$ min for STE. The m/z for internal standard, KET, was $m/z = 253$, with a R_t of 2.8 min.

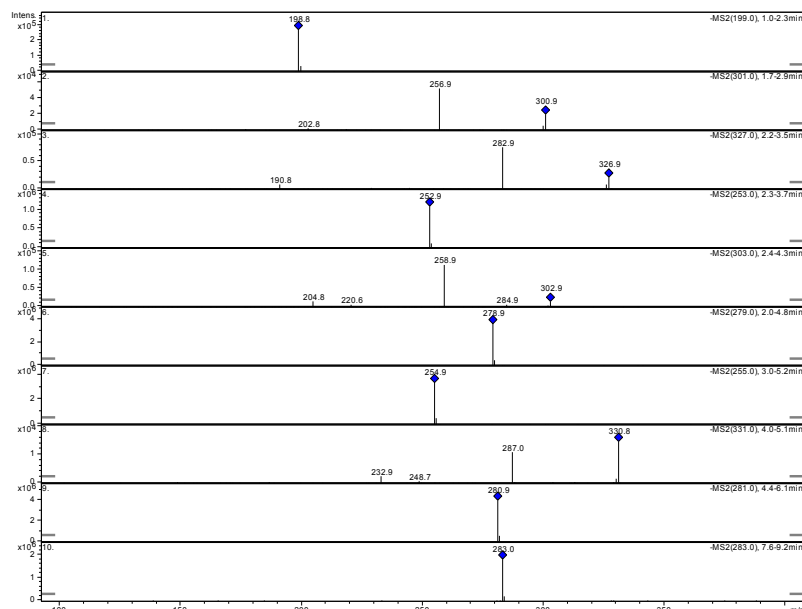


Figure 1.

Product ion spectra of FA ($m/z = 199$ $R_t = 1.4$ min LAU, $m/z = 257/301$ $R_t = 2.0$ min EPA, $m/z = 283/327$ $R_t = 2.5$ min DHA, $m/z = 259/303$ $R_t = 2.7$ min AA, $m/z = 279$ $R_t = 3.1$ min LIN, $m/z = 255$ $R_t = 4.3$ min PAL, $m/z = 287/331$ $R_t = 4.3$ min ADR, $m/z = 281$ $R_t = 4.7$ min OL, $m/z = 283$ $R_t = 8.2$ min STE; internal standard - $m/z = 253$ $R_t = 2.8$ min KET)

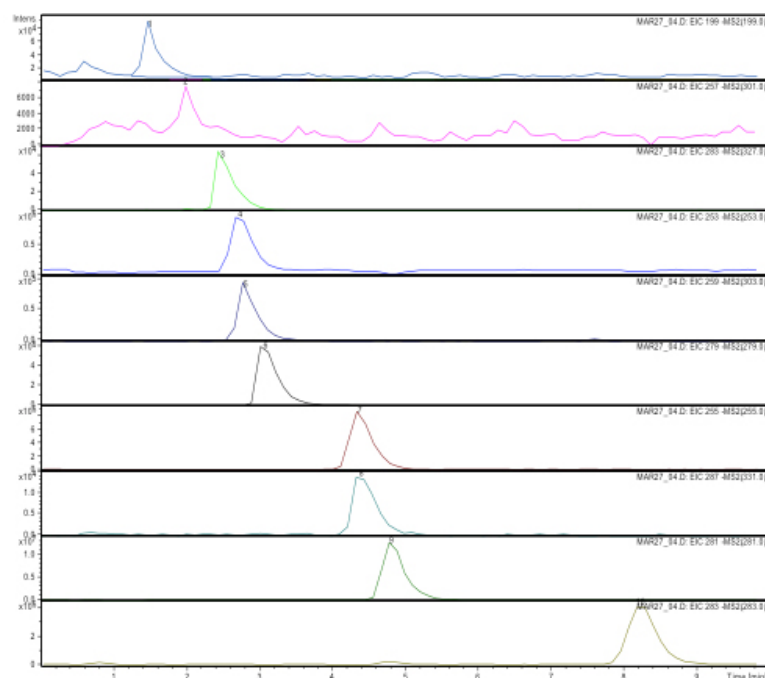


Figure 2.

Chromatogram of a plasma sample (❶ LAU ($R_t = 1.4$ min), ❷ EPA ($R_t = 2.0$ min), ❸ DHA ($R_t = 2.5$ min), ❹ AA ($R_t = 2.7$ min), ❺ KET ($R_t = 2.8$ min), ❻ LIN ($R_t = 3.1$ min), ❼ PAL ($R_t = 4.3$ min), ❽ ADR ($R_t = 4.3$ min), ❾ OL ($R_t = 4.7$ min), ❿ STE ($R_t = 8.2$ min))

To verify whether there is a correlation between plasma levels of certain FA and their incorporation into the RBC membrane, paired plasma – RBC samples from 26 volunteers were analysed.

Results obtained for plasma and RBC membrane levels of the studied FA are presented in Table I. These results show that there is a weak correlation between plasma and RBC membrane levels of LAU ($r = 0.4382$), EPA ($r = 0.5594$) and DHA ($r = 0.5400$), while there is no correlation in case of the other FA.

Table I

Plasma and RBC membrane values of FA

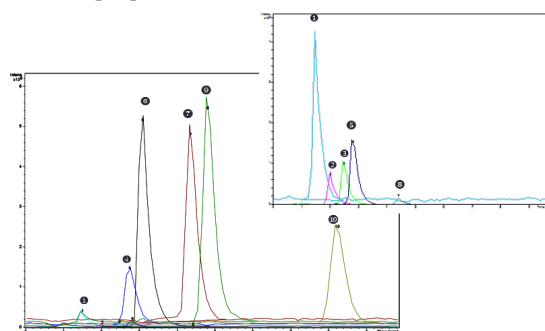
FA	µg/mL Plasma ± SD	µg RBC sample ± SD	r
LAU	1.28 ± 1.63 CV% = 127.70	0.20 ± 0.16 CV% = 78.92	0.4382
EPA	0.05 ± 0.04 CV% = 84.28	1.09 ± 0.91 CV% = 83.95	0.5594
DHA	4.02 ± 2.53 CV% = 62.87	6.82 ± 3.63 CV% = 53.16	0.5400
AA	21.72 ± 13.13 CV% = 60.46	180.98 ± 66.12 CV% = 36.53	0.3259
LIN	29.08 ± 10.09 CV% = 34.69	4.69 ± 1.79 CV% = 38.27	0.4088
PAL	353.64 ± 176.17 CV% = 49.82 %	143.17 ± 52.16 CV% = 36.43%	-0.0300
ADR	0.82 ± 0.69 CV% = 84.32	12.31 ± 4.69 CV% = 38.08	0.3485
OL	721.75 ± 571.29 CV% = 79.15	4.53 ± 1.97 CV% = 43.59	0.3021
STE	116.45 ± 90.15 CV% = 77.41	2.45 ± 1.11 CV% = 45.34	0.1497

Ratios AA/(EPA + DHA), AA/EPA, AA/DHA and Omega-3 Index were calculated and the cumulative results are presented in Table II.

Literature data concerning cardio protective effect of n-3PUFA are controversial - there are numerous epidemiological, observational, multicentre studies, involving a large number of patients, showing that doses between 0.9-1.8 g/day EPA+DHA significantly reduce the risk of sudden cardiac death [9, 21], while other studies contradict reducing cardiovascular morbidity and mortality after chronic treatment with n-3PUFA [22]. However, the anti-inflammatory effect of n-3PUFA cannot be denied as they reduce synthesis of competitive pro-inflammatory molecules from n-6PUFA. A balanced n-3PUFA/n-6PUFA ratio in diet of 1:1 is considered optimal for human body. In Europe this ratio is, in reality, between 1:15 to 1:20 [5].

The effects of n-3PUFA on the heart, such as reducing the arrhythmogenic potential by modifying the function of ion channel, blocking voltage-dependent Na⁺ channel, prolongation of refractory period and increasing membrane potential necessary for depolarization, modulator effect on slow calcium channels, decreasing calcium influx and calcium charging during the ischemic crisis, increasing threshold of ventricular

fibrillation and limiting the damage produced by ischemia, are the subject of numerous studies [4, 10]. Pharmacokinetic data show that n-3PUFA are well absorbed after oral administration [3] and bioavailability is depending on the form of binding (phospholipids > triglycerides > free FA > ethyl esters), the matrix effect (concomitant consumption of foods with high content in fat) or dosage form (emulsion, capsule) [7]. Their incorporation in the membrane phospholipids depends on their concentration in plasma [18]. Another possibility is the increasing of the incorporation of EPA or DHA in the structure of cardiolipin, essential phospholipid for mitochondrial oxidative function of the cardiomyocyte after oral administration of n-3PUFA [20].

**Figure 3.**

Chromatogram of a RBC membrane sample, with detail on 1-5 min interval (1 LAU (Rt = 1.4 min), 2 EPA (Rt = 2.0 min), 3 DHA (Rt = 2.5 min), 4 AA (Rt = 2.7 min), 5 KET (Rt = 2.8 min), 6 LIN (Rt = 3.1 min), 7 PAL (Rt = 4.3 min), 8 ADR (Rt = 4.3 min), 9 OL (Rt = 4.7 min), 10 STE(Rt = 8.2 min))

The results presented in Table II show that in case of AA/(EPA + DHA) ratio there is a correlation between plasma levels and the proportion of these FA in RBC membrane ($r = 0.7807$).

Table II

Parameters value in plasma and RBC membrane

Parameters (N = 26 samples)	Plasma value ± SD	RBC membrane value ± SD	r
AA/(EPA + DHA) ratio	6.14 ± 2.84 CV% = 46.20 %	26.48 ± 10.09 CV% = 38.11 %	0.7807
AA/EPA ratio	656.62 ± 400.07 CV% = 60.92 %	243.42 ± 134.53 CV% = 55.26%	0.3906
AA/DHA ratio	6.21 ± 2.87 CV% = 46.28%	30.07 ± 10.74 CV% = 35.74 %	0.7540
Omega 3 Index	0.36 ± 0.19 CV% = 54.99%	2.22 ± 0.93 CV% = 42.04%	0.4991

If in case of AA/DHA ratio plasma values are significantly higher ($p < 0.001$) than values in the RBC membrane and a good positive correlation between plasma levels and the FA in RBC

membrane is observed ($r = 0.7540$). In the case of AA/EPA ratio plasma values are significantly ($p < 0.001$) lower than in the RBC membrane and correlation between plasma levels of EPA and its levels into the RBC membrane does not exist ($r = 0.3906$), whereas $CV\% > 60\%$ in plasma shows that these values are not homogeneous with very high inter individual variability.

The correlation between Omega-3 Index values in plasma and RBC is weak ($r = 0.4991$) studies presented in literature use RBC FA values and not

plasma levels to eliminate possible interference caused by recent intake of FA [13].

The Omega-3 Index in RBC membranes reflects the situation of FA balance in the last 80-120 days. As it is well known plasma ratio of different FA depends very much on nutrition - the value can be influenced in the same direction by increasing the counter or decreasing the denominator of the fraction, but with different clinical significance, or by other associated pathologies involving lipolysis.

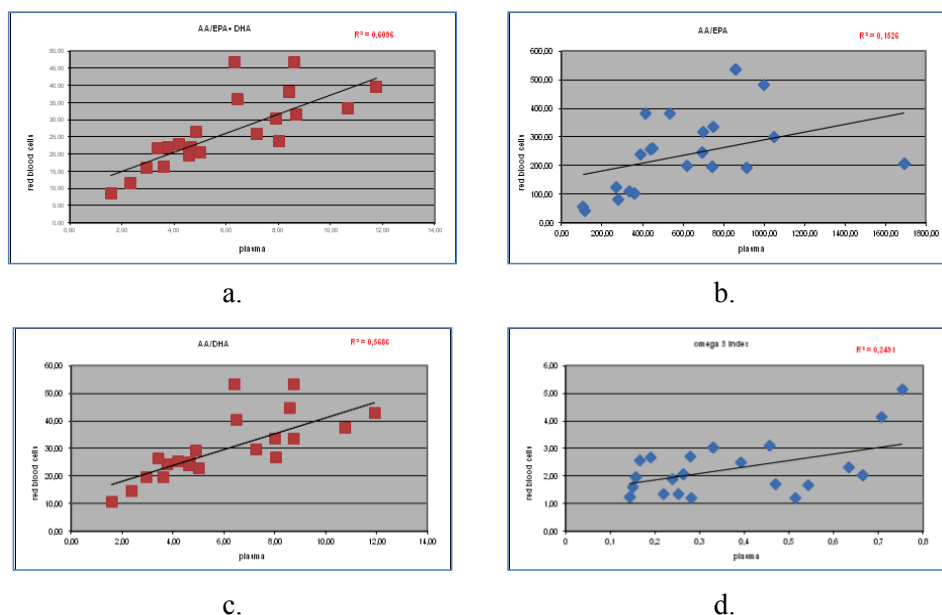


Figure 4.

a. AA/(EPA + DHA) ratio plasma versus RBC, b. AA/EPA ratio plasma versus RBC, c. AA/DHA ratio plasma versus RBC, d. Omega-3 Index plasma versus RBC

Using the Omega-3 Index in evaluation of FA balance in the body is preferred due to the fact that plasma levels indicate recent intake – same as Hb A1c vs glycaemia in diabetic patients. Previous studies showed that EPA+DHA levels in RBC membranes are highly correlated with the ones in the cardiomyocyte [11], on a lower variability compared with plasma and, not least, the easiness of sample obtaining.

In literature there are various methods for the determination of Omega-3 Index. A 2014 meta-analysis showed that by analysing the same sample in 5 different laboratories, very different results were obtained (variation between Omega-3 Index values being 3.5 units) [17]. These variations are due to applied analytical methods (GC/MS or LC/MS) but also due to the number of FA taken into consideration. Although the Omega-3 Index value depends on the type and the number of FA taken into account, however, using the same method in the same laboratory allows monitorization of patient evolution, especially after administration of various pharmaceutical forms of n-3PUFA.

Conclusions

Measuring the Omega-3 Index using a LC/MS method in our study showed significant differences between plasma concentrations and RBC membrane values. There is a positive correlation between the AA/DHA and AA/(EPA + DHA) ratio in plasma and RBC membranes but no correlation between plasma levels of AA/EPA or Omega-3 Index in RBC membranes.

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